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The effect of tumour progression on lymphocyte responses to lectins, measured by fluorescence polarization techniques

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Abstract. The process of fluorochromasia involves the hydrolysis by cells of fluorescein diacetate resulting in an intracellular accumulation of fluorescein. The polarization of the fluorescence of the fluorescein appears to depend on the intracellular fluorescein concentration, the distribution of fluorescein within the cell and the viscosity of the cell cytoplasm.

The parameters of fluorochromasia were studied with thymocytes from normal BALB/c mice and from mice bearing an intraperitoneal NK/LY/R lymphoma. During the course of tumour proliferation, the response to *T*-cell mitogens increased whereas the response to other lectins, *e.g.* wheat germ agglutinin, decreased or remained unaltered. These changes were consistent with the corresponding increase in immunocompetent cells within the thymus, observed by microelectrophoresis. Thus this sensitive technique provides a useful quantitative assessment of the lectin-lymphoid cell interaction.

Keywords. Fluorescein; polarization; lymphocytes; fluorescein fluorescence polarization; fluorochromasia.

Introduction

Fluorochromasia in lymphoid cells, first described by Rotman and Papermaster (1966), involves the intracellular hydrolysis of the non-fluorescent substrate fluorescein diacetate (FDA) to which the cell membrane is permeable forming free fluorescence fluorescein. Since the membrane is less permeable to the reaction product, the net result is an intracellular accumulation of fluorescein increasing with the duration of the reaction. Measurement of fluorescence polarization during fluorochromasia was first carried out by Burns (1969), using yeast cells of *Euglena gracillis* cells. The microviscosity of the cell interior was estimated, and the effects of phenethyl alcohol on yeast cells were determined (Burns, 1971). Cercek and Cercek (1972a, b; 1973) also measured fluorescein fluorescence polarization in yeast cells, subsequently using the technique to make similar measurements on Chinese Hamster ovary cells (Cercek *et al.,* 1973) and human lymphocytes (Cercek *et al.,* 1974). They were able to measure a decrease in the polarization of fluorescence caused by *in vitro* incubation of human peripheral blood lympho-

Abbreviations used: FDA, Fluorescein diacetate; WGA, wheat germ agglutinin; Con A, concanavalin A; PBS, 0.15M phosphate buffer, pH 7.3 containing 0.15 M sodium chloride; PWD, Poke Weed Mitogen.

cytes with phytohaemagglutin (PHA). However, no response to PHA of lymphocytes from patients with cancer could be demonstrated. The response of normal human lymphocytes to PHA as measured by fluorochromasia has been confirmed by many other groups (Dickinson *et al.,* 1976; Kreutzmann *et al.,*1978; Pritchard and Sutherland, 1978; Nishifuku *et al.,* 1977; Takaku *et al.,* 1977; Stewart *et al.,* 1979; Balding *et al.,* 1980 Mitchell *et al.,* 1980; Orjasaeter *et al.,*1979) other lectins, *e.g.* concanavalin A (Con A) or wheat germ agglutinin (WGA) have also been shown to alter polarization of fluorescence in lymphoid cells (Balding *et al.,*1978, 1981). In addition, PHA induced changes in the rate of FDA hydrolysis and leakage of fluorescein from the cells could be measured (Balding *et al.,* 1980, 1981).

The level of the response to PHA of lymphocytes from patients with cancer is still controversial. A number of groups have found similar responses to those of lymphocytes from healthy donors (Dickinson *et al.,* 1976; Orjasaeter *et al.,* 1979; Balding *et al.*, 1980; Mitchell *et al.,* 1980), while others have confirmed the lack of response reported by the Cerceks (Takaku *et al.,* 1977; Kreutzmann *et al.,* 1978; Pritchard and Sutherland, 1978). A cancer-related change in the density of *T*-lymphocytes has been suggested (Pritchard *et al.,* 1978), whereas Orjasaeter *et al.* (1979) suggest that a qualitative change, unrelated to cell density, has occurred in the *T*-lymphocyte population.

In order to gain information about the relationship of changes in the *T*-lymphocyte population during the growth of a tumour and the differing response of such lymphocytes to lectins during fluorochromasia, we have used thymocytes and spleen lymphocytes from normal BALB/c mice and from mice bearing an intraperitoneal ascites lymphoma as a model system. Changes in polarization of fluorescence, rate of FDA hydrolysis and leakage of fluorescin from the cell resulting from incubation of the lymphocytes with several different lectins are repotted.

Materials and methods

Preparation of lymphocyte suspensions was essentially as previously described (Balding *et al.,* 1980). Briefly, human lymphocytes from healthy donors were isolated on Lymphoprep^{R} (Nyegaard and Co., Oslo, Sweden, density 1.079 g/ml) at 15°C after carbonyl-iron treatment of blood samples. Murine thymocytes and spleen lymphocytes were isolated from homogenates of excised thymus or spleen tissue obtained from 6-10 weeks old healthy male inbred BALB/c mice, or from similar mice in which an intraperitoneal NK/LY/R ascites lymphoma had been cultured for the appropriate length of time. Erythrocytes in the spleen but not thymus cell suspensions were lyzed by treatment with 5 mM Tris buffer 0.83% ammonium chloride solution, pH 7.3.

Lymphocytes were washed twice in medium 199 with Earles salts, 25 mM HEPES and *L*-glutamine (Gibco Biocult) and twice in 0.15 M phosphate buffer, pH 7.3 containing 0.15 M sodium chloride (PBS) (0.45 μ Millipore filtered complete Dulbecco phosphate buffered saline, pH 7.3; from Oxoid Ltd., code SR38 plus Code BRl4a). Cells were resuspended in PBS at a concentration of 8×10^{6} (murine lymphocytes) or $4\times l0^{6}$ (human lymphocytes). Cell suspensions were equilibrated at 37° C before addition of 0.1 ml of a saline solution of lectin [PHA: Wellcome purified grade HA16, wheat germ agglutinin (WGA), and Poke Weed Mitogen (PWM), from Sigma London Chemical Co. Ltd.] or 0.1 ml of sterile saline solution to control samples.

Fluorescence and fluorescence polarization measurements were carried out as described previously (Balding *et al.,* 1978, 1980, 1981) using a Perkin Elmer MPF4 spectrofluorimeter. The data was collected using a purpose built analogue to digital converter and analysed by computor (Data General Nova) to avoid subjective bias. Polarization of fluorescence was calculated using the simple formula developed by Perrin (1929), $p= Iv–Ih/Iv+Ih$, where *Iv* is the vertical component and *Ih* the horizontal component of intracellular fluorescence. Total fluorescence intensity *Ft* and extracellular fluorescence intensity *Fs* were calculated from the basic equation, $F=Iv+2Ih–Fo$, where *Fo* is the contribution to fluorescence intensity from spontaneous hydrolysis of FDA substrate measured after an appropriate time using substrate solution without added cell suspension. Inttacellular fluorescence $Fi=Ft–Fs$.

Fluorescence and fluorescence polarization measurements were carried out at 27°C, after 30 min incubation with lectin or saline at 37°C.

Results

An immediate increase in fluorescent intensity was observed after addition of lymphocytes to FDA. The rate of hydrolysis of EDA to fluorescein by human lymphocytes was almost linear, but the rate of accumulation of intracellular fluorescein decreased to a plateau after about 14 min; the polarization of fluorescence tends towards a plateau simultaneously with the intracellular fluorescence (figure 1). During the course of the reaction there was some leakage of fluorescein out of the cells, increasing from about 10% to about 50% of the total fluorescence intensity after 20 min. Polarization of values were therefore compared at equivalent intracellular fluorescein concentration, rather than at equivalent duration of hydrolysis as is usually reported (Cercek *et al.,* 1973, 1978).

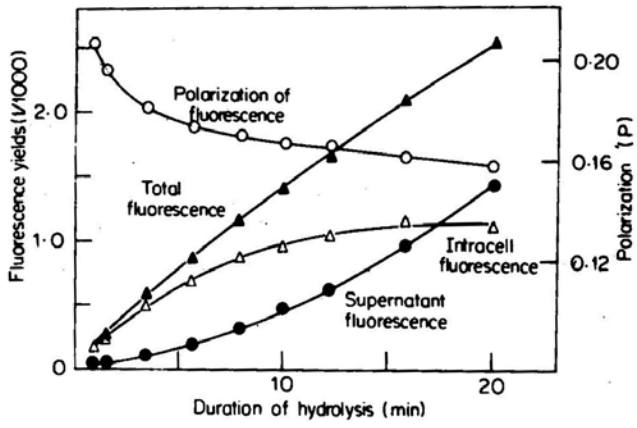

Figure 1. Fluorochromasia in human lymphocytes and the relationship between polarization of fluorescence and duration of FDA hydrolysis at 27° C (Abscissa-arbitrary units 1000 *u*=8 nM NaF).

When lymphocytes were incubated with increasing concentrations of PHA a typical saturation curve was obtained for human lymphocytes and murine spleen lymphocytes (figure 2). Murine thymocytes from control animals, on the other hand did not show any change in polarization value on incubation with PHA.

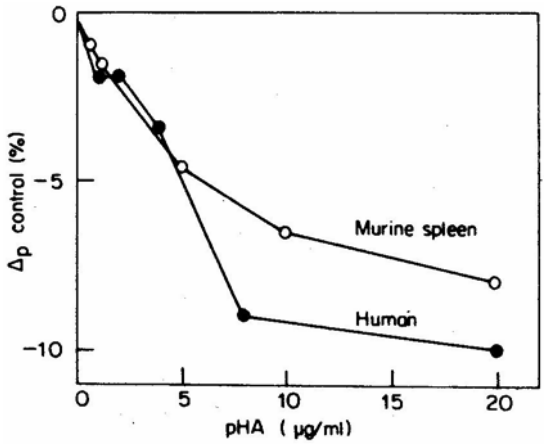

Figure 2. Change in polarization of fluorescein fluorescence for human and murine spleen lymphocytes incubated with PHA.

However, if the thymocytes were prepared from a mouse bearing a 3-day old NK/LY/R ascites lymphoma a similar saturation curve as observed for normal spleen cells was obtained (figure 3). In contrast, thymocytes from the control animal gave a decrease in polarization value after incubation with WGA. Thymocytes from the tumour-bearer showed a much reduced response or a slight increase in polarization value after incubation with WGA.

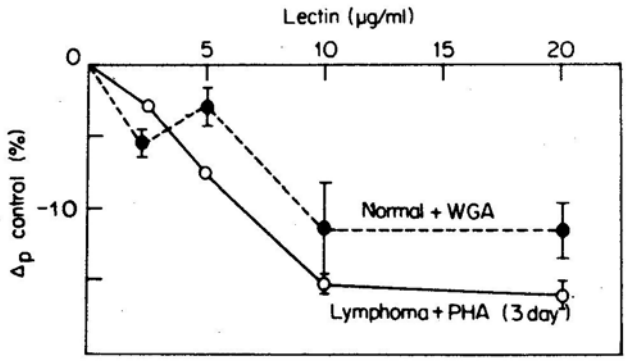

Figure 3. Change in polarization of fluorescein fluorescence for murine thymocytes from control and NK/LY/Rlymphoma bearing animals, incubated with WGA or PHA (± s.e.).

A similar decrease in polarization of fluorescence as was obtained with PHA, was observed after incubation of thymocytes from the tumour-bearing animal with PWM (figure 4), although the response appeared to have dose dependent characteristics, reducing as the concentration of PWM increased. Thymocytes from the control animal showed a marked increase in polarization value at low

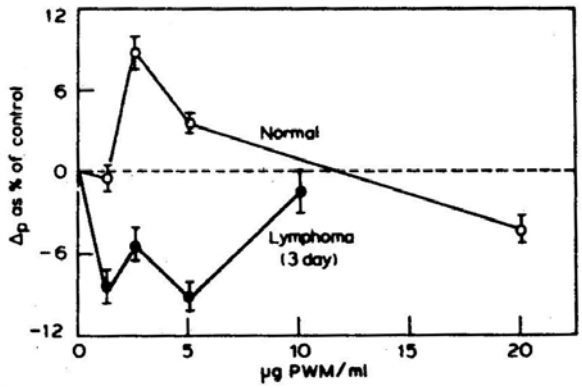

Figure 4. Change in polarization of fluorescein fluorescence for thymocytes from control and lymphoma-bearing mice, incubated with PWM (± s.e.).

concentration, reducing to a slight decrease in polarization value at higher concentrations. In addition, changes in the rate of FDA hydrolysis and leakage of fluorescein from the cells were also observed as shown in figures 5 and 6. An increase in FDA hydrolysis rate of up to 50% was observed with control thymocytes, whereas with thymocytes from the tumour bearing animal the response to PWM was the

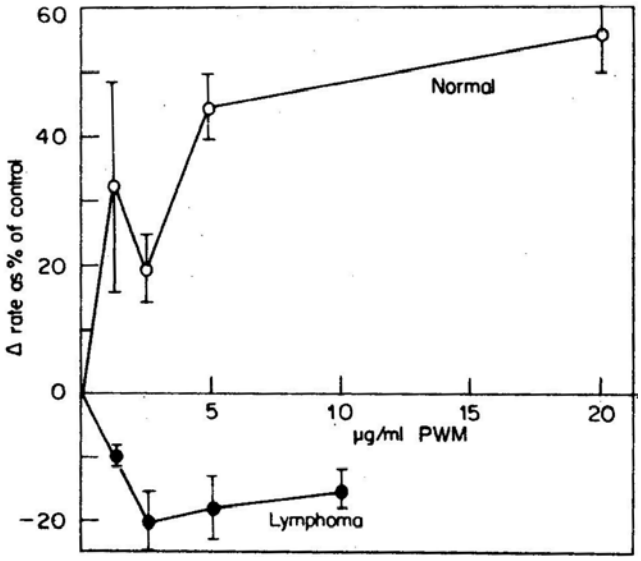

Figure 5. Effect of PWM concentration on the mean rate of FDA hydrolysis for thymocytes from normal and lymphoma bearing mice (± s.e.).

complete converse, *i.e.* a decrease in rate up to 20%. Similarly as shown in figure 6, an increase in the intracellular fluorescein concentration in normal thymocytes was observed, compared to a decrease in intracellular fluorescein level in thymocytes from the tumour bearing animal The intracellular fluorescein concentration or *Fi,* was determined at equivalent total fluorescence levels, and therefore corresponds to a significant change in the rate of leakage of fluorescein out of the cell.

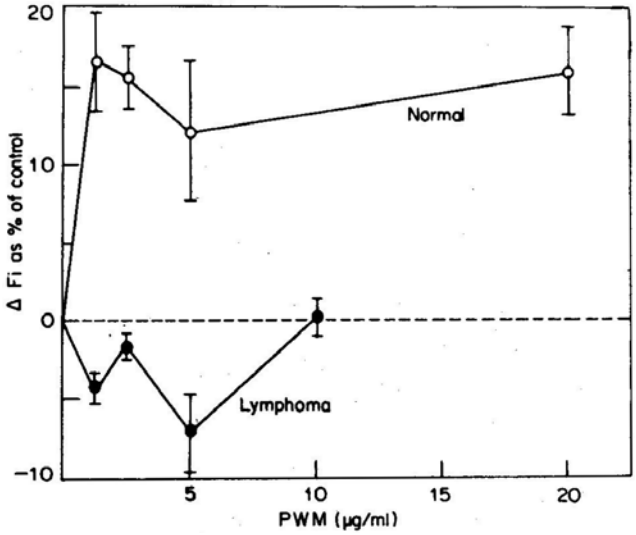

Figure 6. Change in intracellular fluorescein concentration (*Fi*) in thymocytes from normal and lymphoma-bearing mice, incubated with PWM (± s.e.).

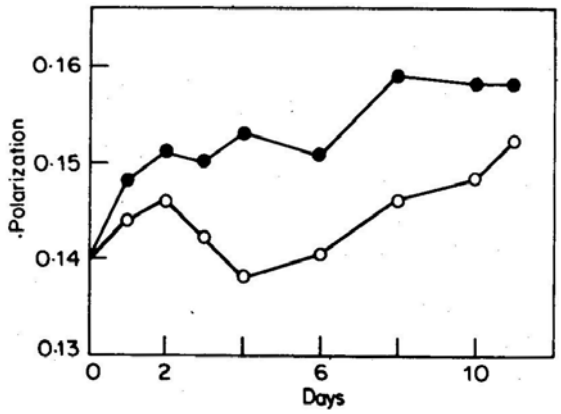

Figure 7. Change in polarization of fluorescein fluorescence in murine thymocytes during the growth of an intraperitoneal NK/LY/R lymphoma. (O), Control; (●),after incubation with PHA at a concentration of 10 μg/8×10^6 cells/ml.

The above results were obtained using thymocytes from mice bearing a 3-day old lymphoma. Measurements were also made using thymocytes prepared from mice bearing a lymphoma for only 24 h and up to 11 days as shown in figure 7. The absolute polarization value increased from 0.14 to 0.16 as the tumour progressed, whereas the response to PGA, *i.e.* the reduction in polarization value, reached a maximum value of about 15% after about 3 to 4 days, gradually decreasing as the tumour progressed.

Discussion

Each of the cell samples studied showed a change in polarization of fluorescence with time, reaching a plateau at higher concentrations of fluorescein, whilst simul-

taneously the rate of accumulation of intracellular fluorescein appeared to be decreasing while the actual hydrolysis rate remained almost linear. It is tempting to ascribe this result to a concentration depolarization effect (Dale and Bauer, 1971). The distribution of fluorescein within the cell is a matter of conjecture, not accurately known, since calculations based on known (or extrapolated) fluorescein concentrations take into account only intra- and extracellular volumes and ignore interaction between fluorescein and membranes or enzymes sites. It is likely, therefore, that any calculation of fluorescein concentration will underestimate the actual local concentrations. Two hypotheses may be considered to explain the observed change in polarization value with time. One possibility is that fluorescein, as formed, is redistributed from a more rigid to a more aqueous phase thus altering the average observed polarization value. This mechanism is supported by single-cell measurements using the cytofluorograph (Udkoff and Norman, 1979). A second possibility is that fluorescein may achieve sufficiently high local concentration for quenching to be significant, with associated depolarization.

The rate of fluorescein production appears to be linar (figure 1). A saturation curve for fluorescein within the cell is also shown. The limiting value of fluorescence may arise when the rate of diffusion of fluorescein out of the cell approaches its rate of production. It is possible that FDA transport into the cell is competitively blocked by fluorescein, if both have affinity for the same structure involved in membrane transport (Steen and Lindmo, 1976). The apparent alteration in intracellular accumulation of fluorescein could be due to feedback inhibition by the reaction products. However the total fluorescence was observed to be increasing when intracellular fluorescence was still at a plateau level, suggesting that saturation or quenching may be more important than inhibition of FDA transport.

The fluorochromasia studies described here have failed to reveal a stable polarization value while fluorescence levels are increasing; indeed there is a close link between polarization values and intracellular concentrations of fluorescein which in turn are dependent upon the hydrolysis rate and the leakage of fluorescein out of the cell. The results reported here were obtained using a final FDA substrate concentration, of 2.4×10^{-6}M, with hydrolysis for up to 20 min at 27°C. Similar effects have been observed by others (Epstein *et al.,* 1971; Udkoff and Norman, 1979; Hashimoto *et al.,* 1979; Mitchell *et al.,* 1980) using similar or different measuring systems and FDA substrate concentrations.

The similarity in the response of murine spleen lymphocytes and human peripheral blood lymphocytes after PHA incubation was not unexpected, since the spleen lymphocyte population corresponds to some extent to the lymphocyte population in the mouse perhipheral system. The lack of response of murine thymocytes from normal animals to PHA or PWM and the increasing response of thymocytes from the lymphoma bearing animal seems to correspond to changes in the mature/immature cell ratio (Jenkins, 1975; Dumont, 1973) during tumour development. These changes have been illustrated by measuring the electrophoretic mobility of individual cells in the suspension as shown in figure 8. For the control animal there is a fast/slow population ratio of about 1 to 9, *i.e.* about 10% fast cells. As the tumour progresses, the ratio of fast to slow cells increases, reaching aout 1 to 2 after about 3 days. The fast population has been identified as consisting of immunocompetent thymocytes, which are released to the periphery. It is

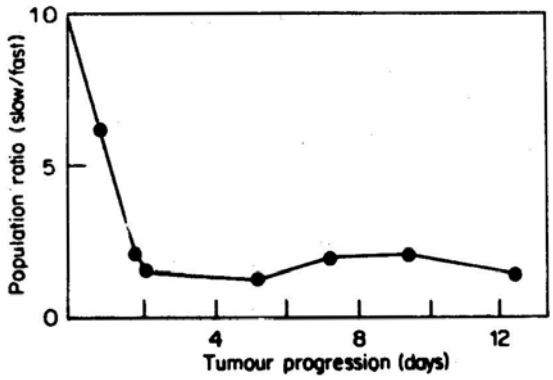

Figure 8. Change in the thymocyte population ratio (slow/fast) during the growth of an intraperitoneal NK/LY/R lymphoma, measured by cell microelectrophoresis.

possibly this population which is responding to PHA and PWM, but not to WGA. Conversely, the immature thymocyte population may show a better response to WGA and other non-mitogenic lectins.

It is therefore possible that it is the immunocompetent *T*-cell population which is responding to PHA in the spleen lymphocyte suspension and by analogy, in the humari peripheral lymphocyte sample.

The thymocyte response to PHA appears to reach a maximum, long before maximum tumour load. It is therefore possible that lymphocytes from a human cancer patient may respond to PHA at one stage of the disease but not at another. Similarly, a significant population of immature lymphocytes or immunoblasts, in the peripheral circulation could lead to an abrogated response to PHA. In the early studies of many cancer detection tests, based on isolated peripheral blood lymphocytes, it is often common practice to compare the response of lymphocytes from young healthy laboratory staff with those from patients with confirmed disease, patients who are often old and in terminal care. The change in the responsiveness of murine thymocytes to PHA during the progression of the tumour lends support to the view that this is bad practice. The discrepancy between the reported results of polarization changes in lymphocytes from patients with cancer could also be related to this observation.

The mechanism of the response of lymphocytes to mitogenic lectins, as detected during fluorochromasia is still unclear. Experiments are in progress to determine whether the assay may be a quick, sensitive and non-subjective physical technique for measuring the early events of lymphocyte activation.

Acknowledgement

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